National Institute of Standards & Technology

# Certificate of Analysis

## Standard Reference Material® 2394

### Heteroplasmic Mitochondrial DNA Mutation Detection Standard

This Standard Reference Material (SRM) is composed of human mitochondrial DNA mixtures which simulate different levels of heteroplasmy. SRM 2394 is intended to provide quality control benchmarks for forensic, medical, and DNA scientists to assess the detection sensitivity of low-frequency mutations, single nucleotide polymorphisms (SNPs) in either mitochondrial DNA (mtDNA) or in pooled nuclear DNA samples, or heteroplasmic sites in mtDNA. SRM 2394 is packaged in a single protective plastic box containing ten tubes: one tube containing the 100 % (by mass) polymorphic DNA, one tube containing the 100 % (by mass) CRS[1] DNA, and eight tubes containing different mass percentages of the polymorphic/CRS mtDNA mixtures (mass % polymorphic levels are 1 %, 2.5 %, 5 %, 10 %, 20 %, 30 %, 40 % and 50 %) (Table 1). Each vial contains 25 µL of DNA at a concentration of 8 ng/µL in 10 mM Tris-HCl, pH 8.5.

The DNA mixtures are constructed from the polymerase chain reaction (PCR) products from two different cell culture lines (CHR and GM09947A) that differ by one base pair (bp) at nucleotide position (np) 6371 in the 285 bp amplified region. The cell line (CHR) designated polymorphic has a T at np 6371 and the cell line (GM09947A) containing the CRS sequence has a C at that site.

**Certified Values:** Table 1 contains the certified values of polymorphic/CRS mtDNA mixtures. The uncertainty values calculated on the 50/50 mixture are reported in section, "Uncertainty Analysis". Table 2 contains the certified sequence information of the 285 bp amplicon from the two cell lines (CHR and GM09947A) and shows the one bp difference at np 6371. The entire 16,569 bp of mtDNA from both CHR and GM09947A was sequenced for SRM 2392, and that sequence can be found in reference 1, the Certificate of Analysis for SRM 2392 [2], and in NIST SP260-155 [3]. Table 3 contains the reference sequences of the forward and reverse primers used in the PCR amplifications of the 285 bp DNAs that were blended to construct these mixtures.

**Expiration of Certification:** The certification of this SRM is valid until **30 July 2009**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Instructions for Use"). This certification is nullified if the SRM is damaged, contaminated, or modified.

**Maintenance of SRM Certification:** NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of the technical measurements leading to the certification were performed by D.K. Hancock and B.C. Levin of the NIST Biotechnology Division.

The analytical determination, technical measurements, and analysis of data for the certification of this SRM were performed by D.K. Hancock, L.A. Tully, and B.C. Levin of the NIST Biotechnology Division.

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[1] CRS refers to the Cambridge Reference Sequence [4] and the revised Cambridge Reference Sequence (rCRS) [5].

Consultation on the statistical evaluation of the data was provided by S.D. Leigh of the NIST Statistical and Engineering Division.

The support aspects involved in the issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by B.S. MacDonald of the NIST Measurement Services Division.

**Permissions:** The research using CHR and GM09947A DNA was deemed exempt from the policy of Part 27 of Title 15 of the Code of Federal Regulations by the NIST Institutional Review Board and the Director of the Chemical Science and Technology Laboratory. This work fits into the exemption category described in 15 CFR 27.101(b)(4) which exempts: "Research, involving the collection or study of existing data, documents, pathological specimens, or diagnostic specimens, if, these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects."

**Storage:** Store frozen at a temperature of –20 °C. Do not store in a self-defrosting freezer because the periodic cycling of temperatures may shorten the shelf-life of this SRM.

## INSTRUCTIONS FOR USE

Minimize repeated freezing and thawing of these materials as this might shorten the shelf-life of the SRM. If it is necessary to perform many repeated analyses, the SRM may be thawed and the tube contents aliquoted into sterile tubes that can be kept frozen until use. Thawing can be conducted at refrigerator temperatures, room temperature, or at 37 °C. After thawing, briefly centrifuge the sample tube to spin down any condensate present in the tube cap, and gently mix to obtain a homogeneous solution. The tube manufacturer cautions that the sample tube caps should be tightened snugly, but not over-tightened. DNA concentrations given are nominal values and are NOT intended for use as concentration standards; only the mass concentration ratios are certified.

## SOURCE AND ANALYSIS[2]

**Source of Material:** CHR DNA was prepared in the NIST DNA Technologies Group, Biotechnology Division. GM09947A DNA was prepared by Life Technologies, Inc. (Gaithersburg, MD). (Note: The current vendor of GM09947A is Marligen Biosciences Inc., Ijamsville, MD.) The 285 bp amplicons from CHR and GM09947A and their mixtures were prepared by the NIST DNA Technologies Group, Biotechnology Division.

**NIST Analysis:** PCR was used to amplify the 285 bp area of the mtDNA from cell lines CHR and GM09947A using the primer set shown in Table 3. The PCR products were sequenced with an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). Multiple techniques were examined to identify an optimal way to determine the relative mass concentration of each amplicon. The techniques examined included ultraviolet (UV) absorbance at 254 nm, fluorescence analysis using a number of DNA dyes on different platforms, peptide nucleic acid (PNA), Denaturing Gradient Gel Electrophoresis (DGGE), Denaturing High Performance Liquid Chromatography and oligonucleotide hybridization analysis using the Luminex 100 system. Once the relative mass concentrations of the PCR products were determined, the mixtures were prepared.

[2] Certain commercial equipment, instruments, materials, or companies are identified in this certificate to specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the materials or equipment identified are the best available for this purpose.

Table 1. Certified Values of Polymorphic/CRS mtDNA Mixtures[(a)]

| Component No. | Color Code | A (CHR) (mass %) | B (GM09947A) (mass %) |
|---|---|---|---|
| 1 | Red | 100.0 | 0.0 |
| 2 | White | 0.0 | 100.0 |
| 3 | Pink | 50.0 | 50.0 |
| 4 | Violet | 40.0 | 60.0 |
| 5 | Blue | 30.0 | 70.0 |
| 6 | Blue-green | 20.0 | 80.0 |
| 7 | Green | 10.0 | 90.0 |
| 8 | Yellow-green | 5.0 | 95.0 |
| 9 | Yellow | 2.5 | 97.5 |
| 10 | Orange | 1.0 | 99.0 |

[(a)] Ten color-coded components are included in each kit. These components are labeled with an "A" designating DNA from cell line CHR (polymorphic) and "B" designating DNA from GM09947A (CRS). Components 1 and 2 contain equal mass concentrations of CHR and GM09947A, respectively. The mixtures (components 3 through 10) were prepared by blending appropriate masses of components 1 and 2.

**Uncertainty Analysis:** The certified values listed in Table 1 correspond to means of the mass concentrations ratios. Each certified DNA ratio is assigned a relative expanded uncertainty about the mean, with coverage factor $k = 2$ (95% confidence level), of 1.7 %, calculated by combining a between-method variance with a pooled within-method variance [6,7] following the ISO and NIST Guides [8] and reference 9. Since all mixtures are prepared from the same stock solutions that were adjusted to have equal (1:1) mass concentrations, and no sequential dilutions were performed, the uncertainty reported on the certified DNA ratios is on the actual quotients of the 50/50 mixture of CHR and GM09947A DNA. A consensus mean, across all measured conditions, for such a quotient is 1.002. The 1.7 % relative error ($k = 2$) associated with the number 1.002 is ± 0.017.

Table 2. Certified Sequence Information of the Two Amplicons from CHR and GM09947A[(a)]

| Cell Culture | | Amplicon Sequence |
|---|---|---|
| CHR | 6242 | 5' cgcatctgc tatagtggag gccggagcag gaacaggttg aacagtctac cctcccttag |
| | 6301 | cagggaacta ctcccaccct ggagcctccg tagacctaac catcttctcc ttacacctag |
| | 6361 | caggtgtctc **T**tctatctta ggggccatca atttcatcac aacaattatc aatataaaac |
| | 6421 | cccctgccat aacccaatac caaacgcccc tcttcgtctg atccgtccta atcacagcag |
| | 6481 | tcctacttct cctatctctc ccagtcctag ctgctggcat cactat 3' 6526 |
| GM09947A | 6242 | 5' cgcatctgc tatagtggag gccggagcag gaacaggttg aacagtctac cctcccttag |
| | 6301 | cagggaacta ctcccaccct ggagcctccg tagacctaac catcttctcc ttacacctag |
| | 6361 | caggtgtctc **C**tctatctta ggggccatca atttcatcac aacaattatc aatataaaac |
| | 6421 | cccctgccat aacccaatac caaacgcccc tcttcgtctg atccgtccta atcacagcag |
| | 6481 | tcctacttct cctatctctc ccagtcctag ctgctggcat cactat 3' 6526 |

[(a)] The one base pair difference is at nucleotide position 6371 and is shown capitalized in bold and underlined. This amplified region of GM09947A agrees with the Cambridge Reference Sequence [4] and the revised Cambridge Reference Sequence [5].

Table 3. Primers Used to Amplify the 285 bp Amplicon Region of Interest

| Primer | | Primer Sequence |
|---|---|---|
| Forward | 6242 | 5' CGC ATC TGC TAT AGT GGA GG 3' |
| Reverse | 6526 | 5' ATA GTG ATG CCA GCA GCT AGG 3' |

**Note:** This amplicon is in the Cytochrome C Oxidase I gene.

## REFERENCES


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[3] Levin, B.C.; Hancock, D.K.; Holland, K.A.; Cheng, H.; Richie, K.L.; *Human Mitochondrial DNA - Amplification and Sequencing - Standard Reference Materials - SRM 2392 and SRM 2392-I*; NIST Special Publication SP260-155; U.S. Government Printing Office: Washington, DC NIST, Gaithersburg, MD (2003); available at http://ts.nist.gov/ts/htdocs/230/232/SP_PUBLICATIONS/PUBLICATIONS.HTM (accessed November 2004).

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[8] ISO; G*uide to the Expression of Uncertainty in Measurement*; ISBN 92-67-10188-9, 1st ed.; International Organization for Standardization: Geneva, Switzerland (1993); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs/ (accessed November 2004).

[9] EUROCHEM; *EURACHEM/CITAC Guide CG 4: Quantifying Uncertainty in Analytical Measurement*; ISBN 0-948926-15-5, 2nd ed.; Ellison, S.L.R.; Rosslein, M.; Williams, A., Eds.; Eurachem Secretariat: Teddington, England (UK) (2000); available at http://www.eurachem.ul.pt/index.htm (accessed November 2004).


*Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751, email srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.*